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ARTICLE

Distinct non-inflammatory signature of microglia in post-mortem brain tissue of patients with major depressive disorder

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Abstract

Findings from epidemiological studies, biomarker measurements and animal experiments suggest a role for aberrant immune processes in the pathogenesis of major depressive disorder (MDD). Microglia, the resident immune cells of the brain, are likely to play a key role in these processes. Previous post-mortem studies reported conflicting findings regarding microglial activation and an in-depth profiling of those cells in MDD is lacking. The aim of this study was therefore to characterize the phenotype and function of microglia in MDD. We isolated microglia from post-mortem brain tissue of patients with MDD ($n = 13$–19) and control donors ($n = 12$–25). Using flow cytometry and quantitative Polymerase Chain Reaction (qPCR), we measured protein and mRNA levels of a panel of microglial markers across four different brain regions (medial frontal gyrus, superior temporal gyrus, thalamus, and subventricular zone). In MDD cases, we found a significant upregulation of *CX3CR1* and *TMEM119* mRNA expression and a downregulation of *CD163* mRNA expression and CD14 protein expression across the four brain regions. Expression levels of microglial activation markers, such as HLA-DRA, *IL6*, and *IL1β*, as well as the inflammatory responses to lipopolysaccharide and dexamethasone were unchanged. Our findings suggest that microglia enhance homeostatic functions in MDD but are not immune activated.

Introduction

Major depressive disorder (MDD) is a common and severe mental disorder that belongs to the ten leading causes of disability worldwide [1]. Several lines of evidence indicate that the immune system plays a role in the pathogenesis of

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MDD [2]: First, systemic IFN-α treatment for hepatitis B and C is associated with a high risk of developing severe forms of MDD [3]. Second, challenging animals with inflammatory triggers leads to depression-like symptoms [4]. Third, infections and autoimmune disorders have been linked to MDD in epidemiological studies [5, 6]. Fourth, increased levels of inflammatory markers have consistently been detected in blood and cerebrospinal fluid (CSF) of MDD patients [7–9]. Finally, positron emission tomography (PET) imaging studies found increased binding of tracers for the translocator protein (TSPO) [8], a marker of neuroinflammation. How the immune system contributes to the pathogenesis of MDD is still unclear, but a role for microglia is hypothesized [10]. Microglia are the resident immune cells of the central nervous system (CNS) and are crucial for the initiation and control of inflammatory responses [11]. Microglia also play important roles during CNS homeostasis and development [12]. They are involved in neurogenesis, axon growth, neuronal survival and synaptic pruning [13, 14]. In addition, they contribute to the metabolism of tryptophan and thereby influence serotoninergic neurotransmission [15].

A dysfunction of microglia is therefore hypothesized to be the connection between the immune system associations found in MDD and the neurobiological changes that underlie the disorder, including alterations in neuroplasticity and neurotransmission [16]. Several post-mortem studies have investigated microglial cells in MDD [8]. These studies focused on signs of microglial activation, including an increased density of microglial cells, changes in microglial morphology, and an upregulation of activation markers (HLA-DR, kynurenine pathway metabolites), but reported conflicting findings (Table S1). Two studies reported increased densities of activated microglial cells [17, 18], whereas seven studies did not find any differences [19–25] and one study reported a decreased density [26]. Changes of microglial morphology towards an activated state was described in three studies [17, 18, 20], but this was not found in one other study [19]. Some studies reported only significant differences in microglial markers for specific regions in the brain, such as the anterior cingulate cortex [17] and hippocampus [26]. Studies focusing on suicide found more evidence for microglial changes [22, 27]. A limitation of these studies is the focus on signs of microglial activation. However, changes in homeostatic properties of microglia, important for brain circuit development and maintenance, may also be relevant for MDD. In addition, previous studies often applied one single methodological approach and used immunostainings with one or two markers, which captures only a few aspects of the complexity of microglia phenotypes [28]. Previous studies also largely varied in brain regions that were analyzed. Microglia phenotype and function are dependent on brain region [29] and an analysis across regions is therefore crucial. Moreover, functional studies on microglia in MDD have not yet been performed.

The goal of this study was to more comprehensively characterize the phenotype and function of microglia in MDD. We isolated microglia rapidly from post-mortem brain tissue (average post-mortem delay 7.5 h), an approach that has been successfully used to characterize phenotypic and functional microglial changes in multiple sclerosis and Alzheimer's disease [30–33]. The first aim of this study was to determine whether these microglia show signs of immune activation or a change in homeostatic markers. We analyzed the expression of a panel of six genes and 16 proteins related to microglia function, including both inflammatory and homeostatic markers. The second aim was to assess whether their inflammatory responses are affected. We analyzed the response of human microglia to the pro-inflammatory stimulus lipopolysaccharide (LPS) and to the glucocorticoid dexamethasone (DEX). To probe for region-specific effects, we included four different regions of interest (ROIs): medial frontal gyrus (FRONT), superior temporal gyrus (TEMP), thalamus (THA), and subventricular zone (SVZ). These regions have also been linked to MDD in imaging studies [34]. In addition, cognitive and mood symptoms of MDD have been related to the frontal and temporal cortex, as well as the limbic system, that includes the thalamus [35]. In addition, we included the SVZ, since this is a human brain region with ongoing neurogenesis in adulthood [36]. Also, the blood brain barrier (BBB) has been shown to be relatively permeable in the SVZ [37], and the tissue is therefore potentially affected by changes in peripheral cytokine levels in MDD [38, 39]. Moreover, we have previously shown differences in microglia phenotype for these four different regions [33, 40].

Materials and methods

Donors

Post-mortem brain tissue of MDD patients and control subjects was obtained from The Netherlands Brain Bank (www.brainbank.nl). The Netherlands Brain Bank (NBB) is a high-quality tissue bank with an exceptional low post-mortem interval (post-mortem delay; median of 7.5 h for the donors in this study). The permission to collect human brain material was obtained from the Ethical Committee of the VU University medical center (VUmc, Amsterdam, The Netherlands). Tissue was collected post-mortem from donors from whom full consent had been obtained during life to conduct brain autopsy and research. We considered including the anterior cingulate, cortex, amygdala and hippocampus as ROIs. However, these structures were not available for a large-scale study with fresh/non-frozen brain tissue.

Table 1 Demographics.

	Controls ($n = 27$)	MDD ($n = 20$)	p-value
Age, mean (SD) in years	81.4 (11.1)	63.2 (22.1)	0.001*
Gender F | M, n	17 | 10	12 | 8	0.836
pH CSF, mean (SD)	6.6 (0.3)	6.6 (0.4)	1.00
PMI, mean (SD) in minutes	448 (116)	470 (145)	0.338
Viable cells, mean (SD) in yield/gram	0.46 (0.4)	0.36 (0.2)	0.310
Smoking, Y | N | NA, n	5 | 16 | 5	7 | 7 | 6	0.163
Alcohol, Y | N | NA, n	11 | 10 | 6	6 | 7 | 7	0.589
Cause of death, n (%)			
Infection	7 (26%)	0 (0%)	0.013*
Cardiorespiratory	5 (18.5%)	3 (15%)	0.750
Cancer	4 (14.8%)	0 (0%)	0.071
Euthanasia	9 (33.3%)	13 (65%)	0.031*
Other	2 (7.4%)	4 (20%)	0.201
Comorbidity, n (Y | N | NA)			
Infection <2 weeks	5 | 22 | 0	2 | 17 | 1	0.502
Autoimmune disease	7 | 20 | 0	5 | 14 | 1	0.296
Neurological disorders	6 | 21 | 0	4 | 15 | 1	0.500
Medication, n (Y | N | NA)			
Benzodiazepines last 24 hours	5 | 17 | 5	4 | 9 | 7	0.598
Opiates last 24 hours	12 | 10 | 5	3 | 10 | 7	0.069
Antidepressants last 3 months	1 | 26 | 1	13 | 6 | 1	<0.001*
Antipsychotics last 3 months	3 | 23 | 1	5 | 14 | 1	0.431
DSM IV diagnosis, n (%)			
Dysthemia	0 (0%)	1 (5%)	
Major depressive disorder, single episode, without psychotic behavior			
Mild	0 (0%)	2 (10%)	
Moderate	0 (0%)	0 (0%)	
Severe	0 (0%)	3 (15%)	
Major depressive disorder, recurrent episode, without psychotic behavior	0 (0%)	1 (5%)	
Mild	0 (0%)	6 (30%)	
Moderate	0 (0%)	6 (30%)	
Severe			
Major depressive disorder, recurrent episode, with psychotic behavior	0 (0%)	1 (5%)	
DSM IV comorbidity, n (%)			
Eating disorder	0 (0%)	2 (10%)	
Adjustment disorder	3 (11.1%)	2 (10%)	
Personality disorder	1 (3.7%)	7 (35%)	
Pain disorder	1 (3.7%)	0 (0%)	
Pervasive development disorder	1 (3.7%)	3 (15%)	
Suicide attempts in history, Y | N | NA, n (%)	1 | 0 | 26	9 | 5 | 5	<0.001*
Tissue, FRONT | TEMP | THA | SVZ, n (donors)[#]	27 | 21 | 21 | 10	19 | 19 | 19 | 16	0.663
Experiments, Protein | Gene | LPS | DEX, n (donors)[#]	16 | 25 | 18 | 12	18 | 19 | 13 | 13	0.682

Other cause of death include ileus, cachexia, dehydration and suicide.

CSF cerebrospinal fluid, *Y* yes, *N* no, *NA* not applicable, *PMI* post-mortem interval, *FRONT* frontal cortex, *TEMP* temporal cortex, *THA* thalamus, *SVZ* subventricular zone, *LPS* 6 hours stimulation lipopolysaccharide, *DEX* 72 hours stimulation dexamethasone.

*p-value <0.05 between patients with MDD and controls based on independent t-test or Pearsons's chi-square test.

[#]Maximum number of donors included per tissue or experiment.

Patients ($n = 20$) are defined as donors with a clinical diagnosis of a major depressive disorder according to the DSM-IV or III. If the DSM-IV or III classification was not available, the DSM characterization was based on retrospective medical chart review by two independent psychiatrists. Control donors ($n = 27$) are defined as donors without a history of a clinical diagnosis of a mood, or psychotic disorder, also confirmed by retrospective medical chart review. We excluded cases that were clinically diagnosed with Alzheimer's or Parkinson disease, amyotrophic lateral sclerosis, Multi System Atrophy, or brain diseases caused by an infection (meningitis and encephalitis) based on retrospective medical chart review. Clinical characteristics of all the included donors are summarized in Table 1, and more extensively in Supplementary Table S2 and per experiment in Supplementary Table S3.

Human primary microglia isolation

An outline of the approach of this study is described in Fig. 1. After autopsy, tissue was stored in Hibernate medium (Thermo Fisher Scientific, USA) on ice. Microglia isolation started as soon as possible, but always within 24 h after autopsy, using a previously described protocol [33, 41]. In summary, fresh post-mortem brain tissue of the four ROIs was mechanically and enzymatically dissociated with collagenase (3700 units/mL; Worthington, USA) and DNase (200 µg/mL; Roche, Switzerland) (FRONT, TEMP, THA) or 0.2% trypsin and 30 mg DNase (SVZ). A Percoll (Amersham, Merck,

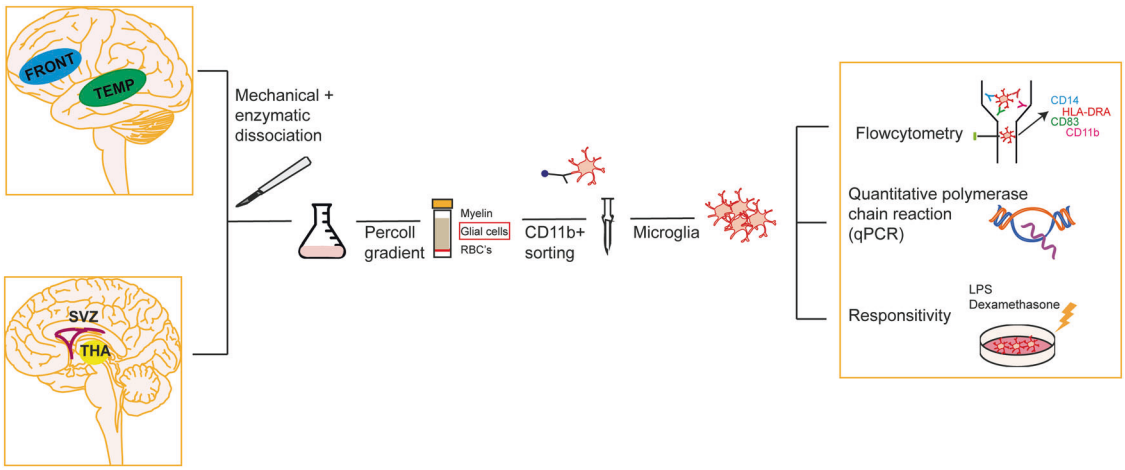
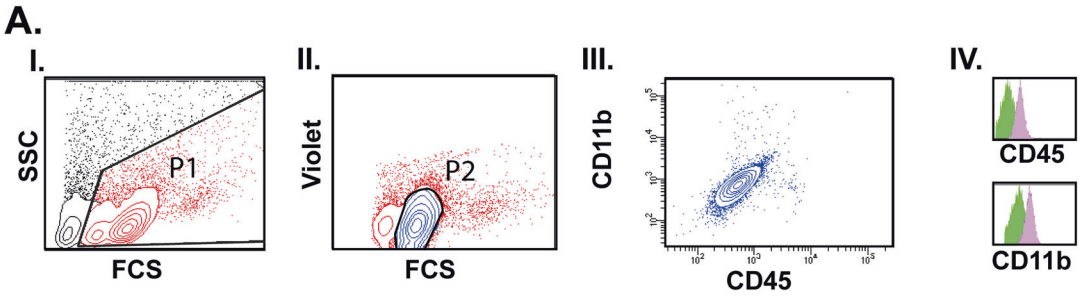


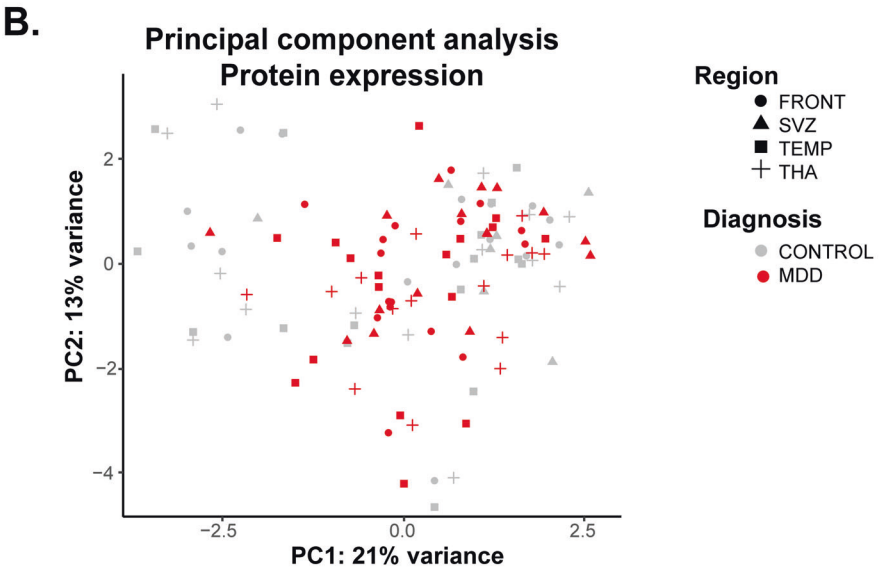
Fig. 1 Outline of methods. Microglia were isolated from the medial frontal gyrus (FRONT), superior temporal gyrus (TEMP), thalamus (THA) or subventricular zone (SVZ). A single cell suspension was generated using a mechanical dissociation step followed by an enzymatic digestion (using DNase, collagenase or trypsin). The glial cell fraction was subsequently extracted using a Percoll gradient and this was followed by purification of $CD11b^+$ cells using magnetic beads. $CD11b^+$ cells (microglia) were eluted and RNA was isolated and stored for quantitative polymerase chain reaction (qPCR). Microglial cells were stained with antibodies and protein expression was quantified using flow cytometry. The cells were also cultured to analyze their response to lipopolysaccharide (LPS) or dexamethasone (DEX).

Germany) gradient was generated to separate viable cells from myelin and cellular debris. The middle layer enriched for microglia was washed twice and myeloid cells were positively selected with CD11b-conjugated magnetic beads (Miltenyi Biotec, Germany). CD11b is also present on perivascular macrophages in the CNS. However, we have previously shown by mass cytometry that the percentage of macrophages ($CD206^{high}$) was low [40]. Moreover, we confirmed in a subsample ($n = 44$ samples derived from 13 donors) of our cohort that the percentage of $P2Y12^+CD64^+$ cells is 96%, confirming that we collected a pure population of microglial cells. The phenotype and responsivity of microglia was characterized by flow cytometry (Fig. 2a), qPCR and by stimulating the cells with LPS or dexamethasone as described by us before [41, 42], and in detail in the supplementary methods and Supplementary Tables S4 and S5.

Statistical analysis

Statistical analyses were performed with R-studio (R version 3.5.3) and Graphpad Prism software (version 8). We tested for significant differences between cases and controls following three steps: (1) We used sample size, mean, and standard deviation (SD) to generate the effect size (ES) between MDD patients and controls for each region. Standardized difference in mean and the upper/lower limit of the 95% CI were used to express the ES. To analyze for overall differences across multiple regions, we generated an estimate of the ES by applying a random effects model using the Comprehensive Meta-Analysis (CMA) software developed by Biostat [43]. (2) Since data were non-normally distributed, as shown by the Kolmogorov-Smirnov test, we subsequently applied the Mann-Whitney U test for the outcomes for each region individually and for all samples pooled together. (3) We then used a linear mixed model to control for potential confounders in our analysis across multiple brain regions using the R package limma version 3.38.3. Data were log-transformed and normalized. To account for multiple samples from different regions of the same donor, we modeled the individual as a random effect and added selected covariates. Age was significantly different between patients and controls and significantly associated with the outcomes and was therefore included as covariate. Other potential confounders, such as pH, PMI, and gender were not significantly different between patients and controls and not associated with any of the outcomes. We therefore applied only adjustment for age. Stimulated data were processed in two separate batches. To adjust for these potential batch effects in the data analyses of stimulated data, we added batch (1 or 2) as additional covariate in the linear model. Limma fits the linear model, and runs a Bayesian moderated *t*-test which provides a *p*-value. We reported outcomes as significant for this study if we found consistent significant findings across all three statistical approaches. Additionally, following log-transformation and correction for age as covariate, we performed a principal component analysis (PCA) of protein and gene expression data from microglia with brain regions and diagnosis as a variable. Bonferroni correction for multiple testing was used (i.e., by dividing the α-level



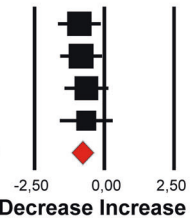


C.

Protein expression

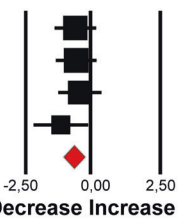
CD14

Brain regions	Effect size	Lower limit	Upper limit	p-Value
FRONT	-0,894	-1,621	-0,167	0,016*
TEMP	-0,832	-1,543	-0,120	0,022*
THA	-0,640	-1,387	0,106	0,093
SVZ	-0,648	-1,556	0,260	0,162
Overall	-0,767	-1,148	-0,385	0,000**



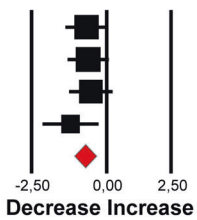
CD64

Brain regions	Effect size	Lower limit	Upper limit	p-Value
FRONT	-0,544	-1,249	0,162	0,131
TEMP	-0,513	-1,207	0,180	0,147
THA	-0,382	-1,117	0,353	0,308
SVZ	-1,062	-2,001	-0,122	0,027*
Overall	-0,575	-0,952	-0,199	0,003**



CD32

Brain regions	Effect size	Lower limit	Upper limit	p-Value
FRONT	-0,720	-1,435	-0,005	0,048*
TEMP	-0,661	-1,362	0,040	0,065
THA	-0,567	-1,309	0,176	0,135
SVZ	-1,294	-2,257	-0,330	0,009*
Overall	-0,752	-1,133	-0,371	0,000**



◀ **Fig. 2 Microglia protein expression profile in major depressive disorder. a** Gating strategy for flow cytometric analysis of human microglia. A representative sample is shown. (I) Cells were first separated from debris using forward scatter and side scatter (P1). (II) These cells were then distinguished from high auto-fluorescent cells using the violet channel as shown in gate P2. The cells in this gate (P2) are positive for CD11b+ and CD45+ as shown in the scatter plots (III) as well as the histograms (IV), where the aspecific staining of the isotype controls is depicted in green. **b** Principal component analysis of protein expression of 117 microglia samples derived from 35 different donors across four different brain regions (FRONT: frontal cortex $n = 32$ donors, TEMP; temporal cortex $n = 33$ donors, THA: thalamus $n = 29$ donors, SVZ; subventricular zone $n = 23$ donors) in MDD cases ($n = 53$ samples derived from 16 donors, red marks) and controls ($n = 64$ samples derived from 18 donors, gray marks). Data were log-transformed and correction for age was applied. The percentage variation in the data explained by the respective principal components is given. Each mark corresponds to an individual sample. **c** Forests plot of CD14, CD32 and CD64 in four different brain regions: medial frontal gyrus (FRONT, controls: $n = 16$, MDD: $n = 16$), superior temporal gyrus (TEMP, controls $n = 16$, MDD: $n = 17$), thalamus (THA, controls: $n = 14$, MDD: $n = 15$), and subventricular zone (SVZ, controls: $n = 7$, $n = 16$). The forest plots show data included in a random effect model, representing effect sizes (standardized difference in means) with 95 confidence interval (CI) for differences between patients with MDD and controls in each brain region separately. The square size is proportional to the sample size. Diamonds at the bottom of the graphs reflect the pooled effect sizes of all brain regions combined (controls $n = 53$ samples derived from 16 donors; MDD $n = 64$ samples derived from 18 donors).

of 0.05 by the number of tested markers per experiment). Subgroup and sensitivity analyses were performed for these significant findings only.

Results

Demographics and microglial numbers

Table 1 summarizes the demographics of subjects included in this study and Supplementary Table S3 provides donor information per experiment. The mean age was significantly different between MDD cases and controls. Other important factors such as sex, pH, post-mortem interval, smoking, and daily alcohol use did not differ between MDD patients and controls. The microglial cell yield (total amount of living cells divided by the grams of tissue) did not differ between MDD cases and controls for individual brain regions (Supplementary Fig. S1) or in total (Table 1).

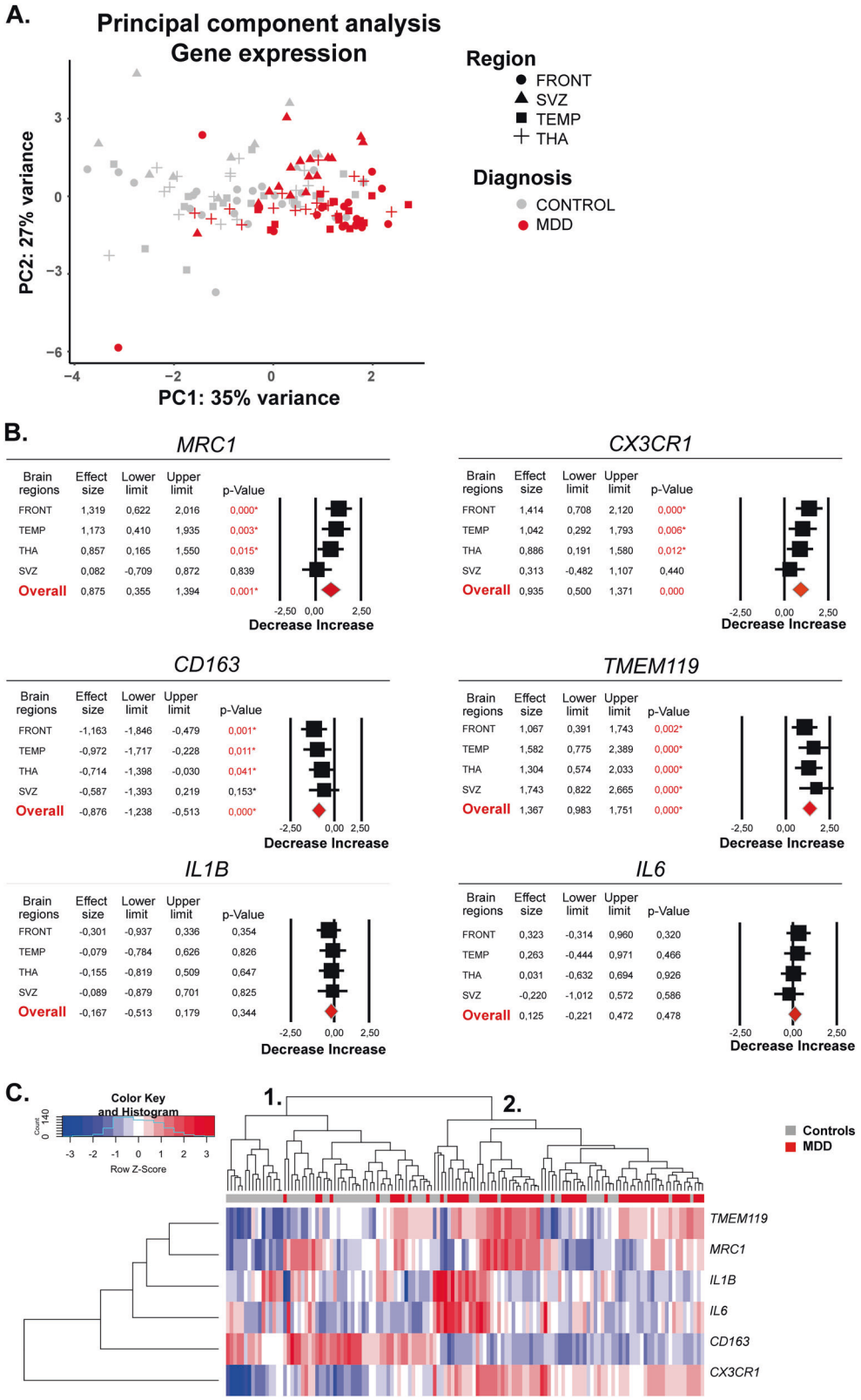
Protein expression levels in microglia from MDD patients and controls

We assessed the expression level of a panel of sixteen proteins by flow cytometry (CD45, CD11b, CD11c, CD14, CD16, CD32, CD40, CD64, and CX3CR1, HLA-DRA, CD163, CD172α, CD200R, CD206, CD83, and CD86) in 53 samples derived from 16 controls and 64 samples derived from 18 MDD patients. The fluorescent mean intensity was variable between donors, suggesting a strong donor-dependent phenotypic variability. A principal component analysis (PCA) of protein expression levels of all 16 markers showed that microglial samples did not segregate based on diagnosis or region (Fig. 2b; Supplementary Fig. S2). We found an overall decreased expression of CD14 (Fig. 2c; Supplementary Fig. S3; Supplementary Table S6). CD64, CD32 were significantly decreased in the random effect model, but lost significance after applying the Mann Whitney U test and linear model (Fig. 2c; Supplementary Table S6). Regional effects were suggested for CD14 in frontal and temporal cortex, CD32 in temporal cortex and SVZ, CD64 in temporal cortex (Supplementary Fig. S3). Other proteins were not significantly different between MDD cases and controls (Supplementary Fig. S4; Supplementary Table S6).

Gene expression levels in microglia from MDD patients and controls

We subsequently analyzed gene expression levels of a panel of microglial markers in MDD cases ($n = 68$ samples derived from 25 donors) and controls ($n = 66$ samples derived from 19 donors). We defined three categories: (1) genes with important homeostatic properties (*TMEM119* and *CX3CR1*) [44–46], (2) genes upregulated upon immune activation (*IL1B* and *IL6)*, and (3) genes upregulated in responses to 'anti-inflammatory' triggers (*CD163* and *MRC1*) [47, 48]. A PCA of expression levels of those 6 selected genes showed that microglial samples segregated between MDD patients and controls (Fig. 3a; Supplementary Fig. S5). Microglial samples of frontal, temporal cortex and thalamus were highly similar, but slightly different from the SVZ samples (Fig. 3a; Supplementary Fig. S5).

We found an overall significant increase for *TMEM119, CX3CR1*, and *MRC1*, and an overall significant decrease in *CD163* between MDD patients and controls (Fig. 3b; Supplementary Fig. S6). We found no differences in the expression of *IL1B* and *IL6* (Fig. 3b; Supplementary Fig. S6). Significance was lost for *MRC1* after applying the linear model (correction for age and multiple brain regions from the same donor; Supplementary Fig. S6A). The observed differences were found in the four brain regions, but less pronounced in the SVZ (Supplementary Fig. S6B). Next, we analyzed the relationship between the different microglial markers: *CX3CR1*, *TMEM119* and *MRC1* correlated significantly with each other (r_s = ranging between 0.47 to 0.50, $p < 0.001$). Both *CX3CR1* and *TMEM119* inversely correlated with *CD163* (r_s = ranging between −0.31 and −0.63, $p < 0.001$). We performed a hierarchical clustering analysis and found that the microglial samples


A.
Principal component analysis
Gene expression
PC2: 27% variance
PC1: 35% variance
Region
FRONT
SVZ
TEMP
THA
Diagnosis
CONTROL
MDD
B.
MRC1
Brain regions | Effect size | Lower limit | Upper limit | p-Value
FRONT 1,319 0,622 2,016 0,000*
TEMP 1,173 0,410 1,935 0,003*
THA 0,857 0,165 1,550 0,015*
SVZ 0,082 -0,709 0,872 0,839
Overall 0,875 0,355 1,394 0,001*
CX3CR1
Brain regions | Effect size | Lower limit | Upper limit | p-Value
FRONT 1,414 0,708 2,120 0,000*
TEMP 1,042 0,292 1,793 0,006*
THA 0,886 0,191 1,580 0,012*
SVZ 0,313 -0,482 1,107 0,440
Overall 0,935 0,500 1,371 0,000
CD163
Brain regions | Effect size | Lower limit | Upper limit | p-Value
FRONT -1,163 -1,846 -0,479 0,001*
TEMP -0,972 -1,717 -0,228 0,011*
THA -0,714 -1,398 -0,030 0,041*
SVZ -0,587 -1,393 0,219 0,153*
Overall -0,876 -1,238 -0,513 0,000*
TMEM119
Brain regions | Effect size | Lower limit | Upper limit | p-Value
FRONT 1,067 0,391 1,743 0,002*
TEMP 1,582 0,775 2,389 0,000*
THA 1,304 0,574 2,033 0,000*
SVZ 1,743 0,822 2,665 0,000*
Overall 1,367 0,983 1,751 0,000*
IL1B
Brain regions | Effect size | Lower limit | Upper limit | p-Value
FRONT -0,301 -0,937 0,336 0,354
TEMP -0,079 -0,784 0,626 0,826
THA -0,155 -0,819 0,509 0,647
SVZ -0,089 -0,879 0,701 0,825
Overall -0,167 -0,513 0,179 0,344
IL6
Brain regions | Effect size | Lower limit | Upper limit | p-Value
FRONT 0,323 -0,314 0,960 0,320
TEMP 0,263 -0,444 0,971 0,466
THA 0,031 -0,632 0,694 0,926
SVZ -0,220 -1,012 0,572 0,586
Overall 0,125 -0,221 0,472 0,478
-2,50 0,00 2,50
Decrease Increase
C.
Color Key and Histogram
Row Z-Score
1.
2.
Controls
MDD
TMEM119
MRC1
IL1B
IL6
CD163
CX3CR1

◀ **Fig. 3 Microglia gene expression profile in major depressive disorder. a** Principal component analysis of mRNA expression of 134 microglia samples derived from 44 different donors across four different brain regions (FRONT: frontal cortex $n = 39$ donors, TEMP: temporal cortex; $n = 34$ donors, THA: thalamus $n = 35$ donors, SVZ; subventricular zone $n = 26$ donors) in MDD cases ($n = 66$ samples derived from 19 donors, red) and controls ($n = 68$ samples derived from 25 donors, gray). Data were log-transformed and correction for age was applied. The percentage variation in the data explained by the respective principal components is given. Each mark corresponds to an individual sample. **b** Forests plots of CX3CR1, TMEM119, CD163, MRC1, IL6 and IL1B in four different brain regions: medial frontal gyrus (FRONT, controls: $n = 22$, MDD: $n = 17$), superior temporal gyrus (TEMP, controls $n = 18$, MDD: $n = 16$), thalamus (THA, controls: $n = 18$, MDD: $n = 17$), and subventricular zone (SVZ, controls: $n = 10$, $n = 17$). The forest plots show data included in a random effect model, representing effect sizes (standardized difference in means) with 95 confidence interval (CI) for differences between patients with MDD and controls in each brain region separately. The square size is proportional to the sample size. Diamonds at the bottom of the graphs reflect the pooled effect sizes of all brain regions combined (controls $n = 68$ samples derived from 25 donors; MDD $n = 66$ samples derived from 19 donors). **c** Heatmap z-score hierarchical clustering based on Euclidean distance of mRNA gene expression computed for six microglial related genes in human primary microglia across four different brain regions in controls ($n = 68$ samples derived from 25 donors, red) and MDD ($n = 66$ samples derived from 19 donors, gray). Data are log-transformed and scaled (+1) for each sample since gene expression levels were non-normally distributed. Z-scores were computed afterwards. Each row represents one of the six genes and each column represents one sample. The color scale reflects the z-score with blue for low expression and red for high expression levels. 1. = cluster 1, 2. = cluster 2.

separated into two clusters (Fig. 3c), namely cluster 1 with higher *CD163* and cluster 2 with higher *TMEM119*/*CX3CR1* expression. Samples from MDD cases were significantly more present in cluster 2 and controls in cluster 1 ($\chi^2 = 23.2$, $p < 0.001$).

Stimulation with LPS and dexamethasone

To determine responsiveness of microglia to external stressors, we challenged the isolated human microglia with LPS (100 ng/mL) for 6 hours or DEX (1 µM) for 72 hours and determined mRNA expression levels of several pro- and anti-inflammatory genes. Microglia responded as expected with increased gene expression of *IL1B, IL6*, and *TNF* after LPS stimulation and increased gene expression of *CD163, CD200R* and *MRC1* after DEX stimulation [42]. The response to LPS and DEX stimulation was not different between MDD patients ($n = 26$ or 33 samples derived from 13 or 18 donors) and controls ($n = 23$ or 24 samples derived from 12 or 13 donors) (Fig. 4; Supplementary Fig. S7).

Subgroup analyses

For significant results in the prior analyses, we performed several subgroup analyses. First of all, we performed two sensitivity analyses in which we compared a more homogenous distinct subgroup of MDD patients and controls, as well as subgroups that we matched for age. In both analyses we found the same increased levels of *CX3CR1* and *TMEM119* mRNA expression and decreased levels of *CD163* mRNA and CD14 protein expression in MDD-derived microglia (Supplementary Figs. S8 and S9). We did not find an effect for severity of MDD symptomatology (data not shown), nor antidepressant use (Supplementary Fig. S10). *CX3CR1* was significantly higher expressed, and *CD163* and CD14 significantly lower expressed in cases that were likely depressed in the period prior to death (Supplementary Fig. S11). Additionally, we analyzed whether antidepressant use had an anti-inflammatory effect on microglia but did not observe this in our LPS stimulation data (Supplementary Fig. S12).

Discussion

By rapid isolation of microglia from post-mortem brain tissue, we were able to perform the first in-depth characterization of the molecular phenotype of microglia in MDD and their responsivity to inflammatory triggers. We found an increased gene expression of the homeostatic markers *CX3CR1* and *TMEM119* and a decreased expression of *CD163* and CD14. This signature was present across all brain regions. We did not find an upregulation of immune activation markers nor differences in the responses of microglia to LPS or dexamethasone.

A distinct microglial signature

Recent transcriptome studies revealed a unique molecular signature of human microglia compared to other brain and immune cells, with for instance high expression of *CX3CR1* and *TMEM119* [47, 49–51]. This microglial signature is dependent on the brain micro-environment, since these features are rapidly lost when microglia are activated or isolated form brain tissue and cultured in vitro [52]. This signature, referred to as a non-inflammatory microglial signature with "homeostatic" properties, is also partly lost with increasing age and in neurodegeneration as observed in brain tissue of patients with Alzheimer's disease and amyotrophic lateral sclerosis [30, 53–58]. In our study *CX3CR1* and *TMEM119* were both upregulated, which is opposite to the direction observed in neurodegenerative disorders [30]. Moreover, *CD163* mRNA and CD14 protein expression were downregulated, while those markers are upregulated in Alzheimer's disease [59, 60]. We did not find evidence for a pro-inflammatory type of microglial activation. This is in line with several other gene expression and immunostaining studies in

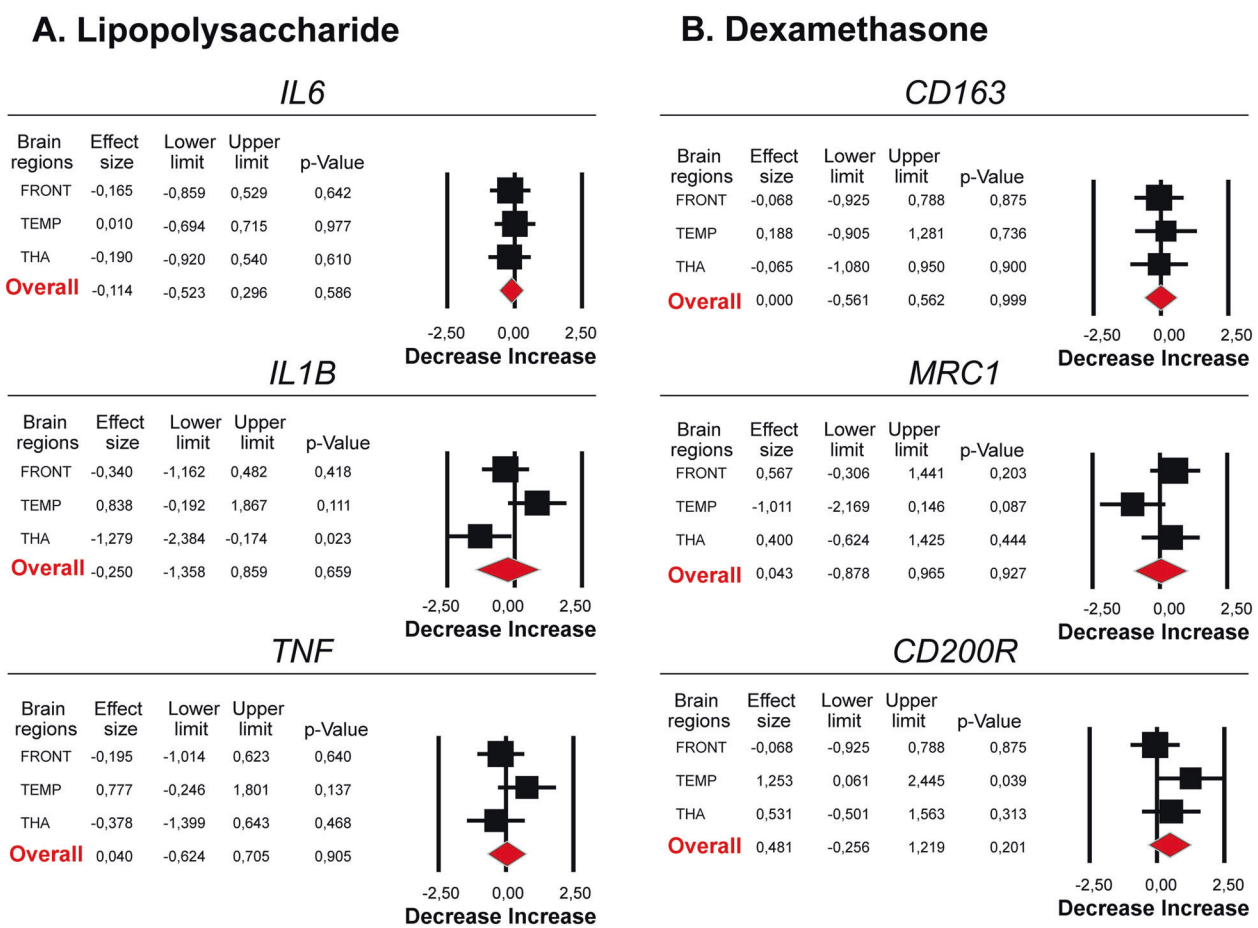


Fig. 4 Microglial responses after stimulation with lipopolysaccharide and dexamethasone. a Forests plot of fold changes of *IL6, IL1B, and TNFA* in three different brain regions: medial frontal gyrus (FRONT, controls: $n = 16$, MDD: $n = 9$), superior temporal gyrus (TEMP, controls $n = 7$, MDD: $n = 9$), thalamus (THA, controls: $n = 10$, MDD: $n = 6$). mRNA expression of *IL1B, IL6*, and *TNF* was first determined by qPCR. Fold changes were calculated by dividing mRNA expression of the LPS-stimulated sample by the mRNA expression of the non-stimulated sample of the same donor. The forest plots show data included in a random effect model, representing effect sizes (standardized difference in means) with 95 confidence interval (CI) for differences between patients with MDD and controls in each brain region separately. The square size is proportional to the sample size. Diamonds at the bottom of the graphs reflect the pooled effect sizes of all brain regions combined (Controls total $n = 33$ samples derived from 18 donors; MDD patients total: $n = 24$ samples derived from 13 donors). **b** Forests plot of fold changes of *CD163, MRC1*, and *CD200R*, in three different brain regions: medial frontal gyrus (FRONT, controls: $n = 10$ donors, MDD: $n = 11$ donors), superior temporal gyrus (TEMP, controls $n = 6$ donors, MDD: $n = 7$ donors), thalamus (THA, controls: $n = 7$ donors, MDD: $n = 8$ donors). mRNA expression of *CD163, MRC1, CD200R* was first determined by qPCR. Fold changes were calculated by dividing mRNA expression of the DEX-stimulated sample by the mRNA expression of the non-stimulated sample of the same donor. The forest plots show data included in a random effect model, representing effect sizes (standardized difference in means) with 95 confidence interval (CI) for differences in fold changes between patients with MDD and controls in each brain region separately. The square size is proportional to the sample size. Diamonds at the bottom of the graphs reflect the pooled effect sizes of all brain regions combined (Controls total $n = 26$ samples derived from 13 donors; MDD patients total: $n = 23$ samples derived from 12 donors).

MDD [19–26, 61]. Previous studies suggested the presence of microglial activation in suicide cases specifically [20, 21, 62, 63]. Only one suicide case was present in our cohort. We did not detect a clear visual different microglial profile in this case. Our findings suggest that microglial cells in MDD patients have a distinct microglial phenotype. It would be interesting to understand how this phenotype relates to binding of the TSPO tracer, a method often used to analyze neuroinflammation in vivo. Binding is increased in several brain regions in MDD [64–67], however, there is more and more debate what type of changes in glial cells are reflected by changes in TSPO binding [68, 69].

Potential consequences of this distinct microglial signature

Microglia play a crucial role in various non-inflammatory processes of the CNS. This includes scavenging, phagocytosis of dead cells and debris, regulation of neurotransmission,

synapse remodeling, and the production of various trophic and synaptogenic factors [11, 13, 14, 70–72]. The shift to a non-inflammatory profile with increased expression of homeostatic markers is noteworthy, since the microglia field has thus far focused more on the consequences of either an inflammatory microglia profile or a loss of homeostatic properties, which is found in neurodegenerative disorders. Hypothetically, these MDD-related microglial changes could result in dysregulation of any of the aforementioned functions, for instance increased activity of synapse remodeling processes. In terms, altered synaptic remodeling could affect MDD-related neuronal circuits. Alternatively, this profile might for instance be a sign of increased microglia motility, which in turn could impact the quality of their scavenging function. Our findings stress the need for more research to understand the role of microglia in non-inflammatory processes in brain disorders.

Potential causes of this distinct microglial signature

Potential factors that have contributed to this altered microglia profile that may be related to causal pathways of the disease are: (A) A depletion of monoamines, including serotonin, norepinephrine, and/or dopamine. Decreased norepinephrine levels were recently associated with a more homeostatic phenotype and function of microglia [73, 74]. (B) A loss of brain-derived neurotrophic factor (BDNF) [75]. BDNF levels have been shown to influence microglia phenotype [76]. (C) The endurance of stress during life. Animal models have linked acute stress to microglial activation, whereas depressive-like symptoms in chronic stress models were associated with hyper-ramification. This hyper-ramification could be a sign of increased homeostatic properties of microglial cells [77–79]. (D) Involvement of TGF-β signaling. Even though genetic associations for MDD cannot be directly related to the expression of any of the differentially expressed microglia genes [80], pathway analysis of MDD-related genes showed a link between MDD and TGF-β signaling [81]. TGF-β is a well-known factor that is important for maintaining the homeostatic profile of microglia [52, 82–84]. (E) In addition to these causal pathways, the microglia signature may have also been triggered by any of the consequences of the disease, such as use of medication or an altered lifestyle. Diet, exercise, sleep and exposure to light can all cause (morphological) changes in microglia [85–89]. Furthermore, selective serotonin and serotonin norepinephrine reuptake inhibitors (SSRI and SNRI, respectively) have been shown to reduce the inflammatory responses of microglia [90–92]. In addition, they are able to induce a ramified morphology and increase *CX3CR1* expression [93–95]. It is not known yet whether antidepressants target the other markers differently expressed in MDD, such as *TMEM119, CD163* and CD14. Although the sample size of our study was too small to formally address this issue, we did not find an impact of antidepressant use during the past three months on the inflammatory responses and the expression of *CX3CR1, CD163, TMEM119*, and CD14 in MDD patients.

Strengths and limitations

Despite the fact that the availability of useful post-mortem tissue is sparse, we were able to include more than 150 microglial samples isolated from fresh brain tissue of 47 different donors. We applied different techniques to elucidate whether microglia are different in MDD. For all experiments, we a priori selected a panel of several microglia-related proteins and genes to investigate differences between MDD patients and controls. A technical limitation is a variable degree of auto-fluorescence which may have confounded the flow cytometry analysis and contributed to a poor correlation between mRNA and protein expression levels. Another limitation of this study is the retrospective phenotyping of the patients and controls. The NBB is known for their comprehensive clinical data files. However, we observed variation in the clinical phenotyping of the MDD cases, and some of the cases in the control group described depressive symptoms during life but have never been clinically diagnosed with MDD. However, our subgroup analysis on patients with the most distinct clinical phenotypes showed similar results. These findings strengthen us in the view that the observed upregulation of *CX3CR1* and *TMEM119* and downregulation of *CD163* and CD14 expression are associated with MDD. Primary microglial changes intrinsic to the illness cannot be distinguished from secondary, compensatory, and epiphenomenal effects by examination of static post-mortem specimens. Furthermore, many ante and peri-mortem factors have a potential impact on microglial phenotype and function, such as antidepressants [96, 97], antipsychotics [98–100], benzodiazepines [101, 102], opiates [103], neurological diseases [104, 105], aging [106], environmental influences and lifestyles, drugs, autoimmune diseases and infections, and cause of death [107]. In this study we reviewed the medical charts of each donor extensively and reported the available information about these potential confounders (as depicted in Table 1 and Supplementary Table S2). However, most factors, such as the use of antidepressants and history of suicide attempts, are highly correlated with the diagnosis of MDD. In addition, the cause of death of the donors was often unclear. Therefore, we were not able to fully adjust for these potential confounding effects in additional analyses. We performed several subgroup analyses, however, certain factors of interest (infection, suicide attempts, autoimmune diseases,

medication use), could not be modeled, due to the small sample sizes in each group. Future larger studies should focus on potential confounding effects. A notable limitation of our microglia isolation protocol is the fact that we sort with CD11b and analyze the cells at the bulk level. A more specific marker for microglia or analysis at the single cell level could strengthen the findings and show whether these are specific to a microglia subpopulation [40].

In summary, the present study provides a characterization of changes in microglia phenotype and responsiveness in MDD. We found a distinct microglial profile, with increased expression of *CX3CR1* and *TMEM119*. These results suggest that microglial functions important for non-inflammatory conditions, such as scavenging, synaptic pruning and neuron-glia interactions, may be affected in MDD. These insights provide information about the potential underlying biological mechanisms in MDD. Functional consequences of these changes and whether they can be targeted for therapy need to be elucidated in future studies.

Supplementary information "Supplementary information is available at MP's website".

Data availability

"Data is available upon request".

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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